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(54) Title: TUBERCULOSIS DRUG TARGETS

(57) Abstract: The present invention relates to a method for identifying compounds capable of affecting the activity of members of a *Mycobacterium* serine/threonine kinase family. A new method of developing drugs for treating *M. tuberculosis* is provided. Unique kinases *(PknB, PknG, PknH, PknJ)* to the organism are used to discover compounds capable of inhibiting them. Knockout mutants of the respective kinases display slower growth and viability both *in vitro* and *in vivo*. These observations are particularly noticeable in the case of *pknG* mutants.

TUBERCULOSIS DRUG TARGETS

BACKGROUND OF THE INVENTION

Field of Invention

The invention relates to the field of drug discovery, in particular within the field of *Mycobacterium tuberculosis* anti-infective agents.

Description of Related Art

Mycobacterium tuberculosis (TB) kills two million people each year. The global epidemic is growing and becoming more dangerous. The breakdown in health services, the spread of HIV/AIDS and the emergence of multidrug-resistant TB are contributing to the worsening impact of this disease. In 1993, the World Health Organization (WHO) took an unprecedented step and declared TB a global emergency. It is estimated that between 2000 and 2020, nearly one billion people will be newly infected, 200 million people will get sick, and 35 million will die from TB.

Nearly one percent of the world's population is newly infected with TB each year. Overall, one-third of the world's population is currently infected with the TB bacillus.

HIV and TB form a lethal combination, each speeding the other's progress. Someone who is HIV-positive and infected with TB is many times more likely to become sick with TB than someone infected with TB who is HIV-negative. TB is a leading cause of death among people who are HIV-positive. It accounts for about 15% of AIDS deaths worldwide

Until about 50 years ago, there were no drugs to cure TB. The development of antibiotics was an enormous boon and many ill patients were successfully treated. Now, however, strains of TB resistant to all major anti-TB drugs have emerged in every nation. A particularly dangerous form of drug-resistant TB is multidrug-resistant TB (MDR-TB), which is defined as disease due to TB bacilli

resistant to at least isoniazid and rifampicin---the two most powerful anti-TB drugs. MDR-TB is rising at alarming rates in some countries, especially in the former Soviet Union, and threatens global TB control efforts.

The *M. tuberculosis* genome has been sequenced. This genome includes a number of proteins including of eukaryotic-like protein kinases and phosphatases that now form the *M. tuberculosis* STPK family (A-Gay Y et al. (2000) Trends in Microbiology 8(5): 238-44). This family is composed of at least eleven protein kinases and four protein phosphatases. Of the eleven predicted serine/threonine kinases, all but two of these proteins contain putative transmembrane domains that suggest they may be localized to the plasma membrane. *M. tuberculosis* STPK family members including *pknD* , *pknB*, *pknI* and *pknG* have been cloned, and shown to encode functional serine/threonine kinases (Peirs, P. et al., (1997) Eur J Biochem. 244(2): 604-12; Av-Gay, Y. et al. (1999) Infection & Immunity 67(11): 5676-82).

SUMMARY OF THE INVENTION

Various embodiments of this invention provide a method of screening for a modulator of a mycobacterium STPK activity comprising: i) contacting a sample containing a mycobacterium STPK with a candidate agent; ii) determining activity of STPK in the sample of (i) subsequent to the contacting; iii) comparing the activity determined in (ii) to activity of the STPK in a sample of (i) without the candidate agent present; wherein, a difference in STPK activity in samples with and without the candidate agent present, is indicative that the candidate agent modulates STPK activity.

Various embodiments of this invention provide an assay for detecting modulation of mycobacterium STPK activity, comprising: i) determining a first STPK activity level in a sample of a mycobacterial STPK; ii) determining a second STPK activity level of a sample of (i) further comprising a candidate agent; iii) comparing the first and second levels; wherein, difference in the first and second levels relates to an ability of the candidate agent to modulate STPK activity.

In the aforementioned embodiments of this invention, the STPK may be one or more of PknG, PknB, PknJ and PknH. Particularly preferred is when the STPK in a method of this invention comprises or is PknG. These methods are useful because a decrease in activity of the STPK, particularly PknG in the presence of a candidate agent is indicative that the agent will decrease growth or survival of mycobacteria, including *M. tuberculosis*.

Various other embodiments of this invention provide a method for assessing a difference in virulence of a candidate mycobacterium as compared to a reference mycobacterium, comprising i) measuring PknG activity in a candidate mycobacterium sample; ii) comparing the activity determined in (i) to PknG activity in a reference mycobacterium sample, where the reference mycobacterium is of known virulence; and wherein, a decrease in PknG activity in the candidate sample is indicative of a decrease in virulence as compared to the reference bacterium, and an increase of PknG activity in the candidate sample is indicative of an increase in virulence as compared to the reference bacterium.

Various other embodiments of this invention provide a method of inhibiting mycobacterium PknG activity or survival of a mycobacterium containing PknG, comprising contacting a sample of PknG or a mycobacterium comprising PknG with an isoquinolinesulfonyl compound or salt thereof. This method may be performed *in vitro*.

Various other embodiments of this invention provide mutant strains of *M. tuberculosis* deficient in one or more STPK selected from: PknG; PknB; PknJ; and PknH. The mutant strain may comprise a mutated STPK gene or a gene incapable or expressing a STPK. Preferably, the strain is deficient in PknG.

Various other embodiments of this invention provide the use of an isoquinolinesulfonyl compound or salt thereof, for preparation of an agent for inhibiting mycobacterium growth or survival. Preferably, the mycobacterium is *M. tuberculosis*. The agent may be a medicament comprising an

isoquinolinesulfonyl compound or salt thereof in combination with a pharmaceutically acceptable carrier.

Other aspects and features of the present invention will become apparent to those ordinarily skilled in the art upon review of the following description of specific embodiments of the invention in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

In drawings which illustrate embodiments of the invention,

Figure 1 is a graphical representation of the inhibition of BCG growth in 7H9 + OADC media by the isoquinolinesulfonyl H7 over a 10 day period. Black squares indicate solvent treatment, black triangles indicate 3mM H7 treatment and empty triangles indicate 0.03 mM hygromycin treatment. Data shown are the means +/- S.E.M. of percent bacterial growth from three separate experiments.

Figure 2 is a graphical representation of the in vitro growth of *M. tuberculosis* H37Rv NCTC 7416 (diamonds), *pknJ* (squares), *pknH*(triangles), and *pknG* ("X") kinase mutants as measured by optical density of cell culture lysates. Slower growth was displayed by *pknJ* and *pknH* mutants. Dramatically slower growth was observed for knockouts of *pknG*. The curves represent an average of multiple growth experiments.

Figure 3 is a graphical representation of *M. tuberculosis* H37Rv growth in various tissues of infected Balb/C mice compared to *pknG* mutant strain. The dashed line is wt and the solid lines represent the data from two separate experiments with *pknG* mutants. The same infectious dose was administered to all animals. *PknG* mutants displayed decreased survival at day 0, and minimal to negative growth at day 30. The X axis represent days and the Y axis represents Log colony forming units (CFU).

Figure 4 is a graphical representation of the survival of SCID mice infected with *M. tuberculosis* H37Rv (squares), *pknH* (triangles), *pknJ* (upside-down triangles) and *pknG* (diamond) infected in terms of percent survival over a period of 50 days. The mutant strains afforded their hosts better survival than the wt bacterial strain (wt infected mice died at 31 days post infection).

Figure 5 is a graphical representation of the data developed in testing bacterial growth in various types of media. Briefly, *M. tuberculosis* H37Rv (open triangle), *pknG* mutant (open square), wt in 7H9 media (solid triangle) and *pknG* mutant in 7H9 media (solid square) are grown over 51 days. The *pknG* mutant in depleted media showed the poorest growth as shown by the optical density of lysate at 600nm.

Figure 6 is a graphical depiction of the survival of bacteria in macrophages infected with *M. tuberculosis* H37Rv and *pknG* mutant strains of *M. tuberculosis*. Colony forming units per ml is shown on the Y axis. The measurement were taken on day 1, 4 and 7. The mutant bacteria was overcome by the macrophage defense systems more effectively by the macrophages than the wt.

Figure 7 Is an schematic illustration of the structural analyses of *M. tuberculosis* STPKs.

DETAILED DESCRIPTION

Definitions:

For the present invention, standard medical, molecular biology, and microbiological definitions are applied to all terms.

Kinase - An enzyme that uses ATP to phosphorylate a substrate; also, in older literature, an enzyme that activates its substrate, e.g. enterokinase.

Mycobacteria tuberculosis (TB) - A highly contagious infection caused by the bacterium called *Mycobacterium tuberculosis*.

Therapeutic agent - any molecule, *e.g.* protein, oligonucleotide, antibody or pharmaceutical, with the capability of altering or impeding the physiological function of STPKs as herein defined.

STPKs- *Mycobacterial* protein serine/threonine kinases, including *PknB, PknG, PknH, PknJ,* and homologs thereof (See Av-Gay et al., 1997 and 2000).

PknG- a *Mycobacterial* protein serine/threonine kinase also referred to as MTB_PKNG or Accession number P96256 (Av-Gay, Y et al., 2000)

PknH- *Mycobacterial* protein serine/threonine kinase also referred to as MTB_PKNH or Accession number QII053 (Av-Gay, Y et al., 2000)

PknJ- *Mycobacterial* protein serine/threonine kinase also referred to as MTB_PKNJ or or Accession number Q10697 (Av-Gay, Y et al., 2000)

PkhB- *Mycobacterial* protein serine/threonine kinase also referred to as MTB_PKNB or Accession number P71584 (Av-Gay, Y et al., 2000)

The *pknG* gene is located in an operon with *glnH*, which encodes an extracellular glutamine-binding protein with a lipid attachment site to anchor it to the cell membrane. Since both *glnH* and *pknG* exist in the same operon, *pknG* expression is likely up-regulated under similar conditions to the genes involved in glutamine metabolism. Transcriptional fusions to the *pknG* gene described in the examples herein show that *pknG* expression is induced under starvation conditions, including stationary phase growth and during incubation in minimal media.

A 30 kDa *PknG* fragment is phosphorylated, similar to many eukaryotic signaling proteins, and also contains a tetratricopeptide (TPR) motif. TPR motifs consist of a degenerate 34-amino acid sequence that is generally present in tandem arrays of 3 to 16 motifs. These motifs occur in a wide variety of proteins present in

bacteria, eukarya, and archea. The 30kDa *PknG* fragment and a 56 kDa *PknG* fragment are now shown to be localized within the cell membrane, in spite of their lack of predicted transmembrane domains. This may indicate a strong association with an *M. tuberculosis* membrane protein or porin, possibly through the TPR motif in the case of the 30 kDa fragment.

In vitro and *in vivo* detection of STPKs in a sample of infected tissue or tissue culture allows for the determination of the virulence of the infective agent, or the presence of *M. tuberculosis*. Mutant strains of TB with damaged or deleted STPKs show decreased survival and virulence *in vitro* and *in vivo*. Mutants lacking pknG are rendered far less harmful in animal models.

In vivo infection by STPK mutant *M. tuberculosis* in animals provides a useful model for the study of the efficacy and mode of action of potential anti-infective or antibiotic agents.

ASSAYS, METHODS AND COMPOSITIONS OF THE INVENTION

The screening methods and assay of this invention involve a comparison of STPK activity in the presence of a candidate agent and comparison of this activity to the STPK activity that would be present under identical or substantially similar conditions in the absence of such a candidate agent. For example, the conditions may be such that but for the presence of the candidate agent, STPK activity is present. A decrease of such activity in the presence of a candidate agent indicates that the candidate agent modulates STPK activity through inhibition of such activity. Conversely, an increase in activity indicates that the candidate agent has an agonist effect.

Samples containing an STPK may be a biological sample, such as a sample derived from a mycobacterium or mycobacterium cell extract. However, the sample may be one or more STPK in purified or partially purified form.

STPK activity may be detected and/or measured through a variety of techniques known in the art for such detection or measurement of kinase activity. One such method involves the presence of a substrate for a STPK, which substrate is known to be affected by the STPK, for example through phosphorylation of the substrate. As is recited in the examples herein, a particular useful substrate is myelin basic protein (MPB).

Detection or measurement of an effect of a STPK on a substrate may be performed by a variety of methods known in the art for determining whether a kinase has had an effect on a substrate. One example is by measuring a state of phosphorylation of the substrate. Various means are known in the art for doing so, including the use of a labeled phosphate and the binding of antibodies to the substrate, which antibodies are capable of differentiating a phosphorylated from an unphosphorylated form.

Alternate methods of measuring STPK activity may involve measuring a change in the STPK indicative of a change in its activity. A change in phosphorylation state of the STPK is such a criteria as is measurement of binding of adenosine triphosphate (ATP) to the STPK.

The methods of this invention may be performed using samples which contain an inhibitor of a STPK such as the isoquinolinesulfonyl compounds (or suitable salts thereof) disclosed herein. For example, the affect of a candidate agent on STPK activity may be compared to a reference sample in which the STPK activity is inhibited by such an inhibitor.

The assay and methods of this invention may be conveniently conducted in various *in vitro* formats. For example, components required in the steps or assay of this invention may be provided on solid substrates. For example, a STPK to be investigated may be bound to a solid substrate. Likewise, a substrate for a STPK may be supported by a solid substrate. Other methodologies are known in the art such as the use of columns, multi-well test plates, etc.

This invention also provides methods for inhibiting PknG and the survival or activity of a mycobacterium containing PknG in which a sample of the kinase, or a mycobacterium is contacted with an isoquinolinesulfonyl compound or acceptable salt thereof.

Antibodies to STPK kinases are also useful for labeling and detection of the kinases in the methods and assay of this invention or for detecting a difference in STPK forms such as a phosphorylated state.

As disclosed herein, mutant strains of *M. tuberculosis* made deficient in one or more STPK are useful in methods of this invention. These mutant strains may be produced, for example as disclosed in the Examples herein, by mutation of the appropriate gene encoding the STPK. Alternate methods exist, including the use of antisense technology to prevent expression of a STPK. Oligonucleotides complementary to STPK coding sequence are also useful in this invention for isolation, labeling, or production of STPK sequences or in the aforementioned antisense technology.

Cloning and Amplification of STPK

Genomic DNA of an organism such as *M. tuberculosis* H37Rv may be prepared by known methods (Av-Gay, Y. et al., 1997 and 2000). Open reading frames are amplified using primers containing restriction sites, and. PCR may be performed with standard reagents and by known methods. PCR products may be separated on gel, ligated into a desired vector and transformed into *E. coli* by standard transformation procedures. Clones containing the vector are selected for on selective media, and plasmid DNA digested and desired DNA isolated.

cDNA preparation and RT-PCR: Expression and purification of STPK

Competent cells of *E. coli* are prepared according to the $CaCl_2$ method (Sambrook, J et al. 1989) and transformed using heat shock. The transformed *E. coli* cells were then plated onto selective media. Colonies are inoculated into

selective media and incubated. Individual cultures isolated and grown, and after centrifugation proteins are separate from pellets by standard techniques.

STPK inclusion bodies contained in the insoluble fractions may be purified from *E. coli* membrane proteins by washing in B-Per reagent and centrifugation. *STPK* is separated by electrophoresis and stained or transferred to membranes. The sequences identity may be checked by standard methods, *eg* Edman degradation and sequence determination as provided, for example at the University of British Columbia Protein Sequencing Laboratory. In order to obtain soluble protein, *STPK* inclusion bodies are resuspended in buffer and added to a urea- dithiothreitol solution. Soluble *STPK* can then be dialyzed, centrifuged, and column purified by size exclusion. The purity of the *STPK* was tested by subjecting samples to SDSPAGE followed by Coomassie blue staining.

Protein Production

Recombinant DNA plasmids bearing restriction enzyme sites such as EcoRI are spliced with the DNA expressing desired protein using known hybridization and ligation methods. The resulting recombinant DNA can be inserted into bacteria, and which produce millions of copies of the specific genetic sequence of interest. The bacterial hosts divides and produces numerous clones of the original transfected cell.

Enzymes and chemicals that cause the bacteria to overexpress the protein encoded by the recombinant plasmid can then be introduced into the media solution containing the bacteria. The end results is large amounts of the desired protein, which is purified by lysing the bacteria either chemically or mechanically, and then purifying the protein by gel-electrophoresis or other suitable means.

Antibodies

Antibodies or portions thereof that are specific to STPK may be useful in assays for the STPKS, in diagnosis of patient samples, or in detecting or visualizing STPKs in various assay situations. As used herein, the term "antibodies"

includes antibodies of any isotype, fragments of antibodies which retain specific binding to antigen, including, but not limited to, Fab, Fv, scFv, and Fd fragments, chimeric antibodies, humanized antibodies, single-chain antibodies, and fusion proteins comprising an antigen-binding portion of an antibody and a non-antibody protein. The antibodies may be detectably labeled, *e.g.*, with a radioisotope, an enzyme which generates a detectable product, a green fluorescent protein, and the like. The antibodies may be further conjugated to other moieties, such as members of specific binding pairs, *e.g.*, biotin (member of biotin-avidin specific binding pair), and the like. The antibodies may also be bound to a solid support, including, but not limited to, polystyrene plates or beads, and the like.

As used herein, a compound which specifically binds to *pknB*, *pknG*, *pknH*, or *pknJ* is any compound (such as an antibody) which has a binding affinity for any naturally occurring isoform, splice variant, or polymorphism. As one of ordinary skill in the art will appreciate, such "specific" binding compounds (*e.g.*, antibodies) may also bind to other closely related proteins which exhibit significant homology, for example, having greater than 90% identity, more preferably greater than 95% identity, and most preferably greater than 99% identity with the amino acid sequence of *pknB*, *pknG*, *pknH*, or *pknJ*.

The ability of the *Mycobacteria* to survive in the presence of therapeutic agents generated by assays of the invention can be tested in various models to ascertain clinical utility. *In vivo* models for *M. tuberculosis* infection are available in mice or rats, and have been described. In these models, the effect of the candidate therapeutic agents on the infection in these models can evaluated, wherein the ability of the therapeutic agents to alter kinase activity is indicated by a decrease in bacterial viability or a reduction in clinical symptoms. Thus, therapeutic agents that exhibit the appropriate anti-infective effect may be selected without direct knowledge of a binding ligand.

Polyclonal antibodies may be raised by a standard protocol by injecting a production animal with an antigenic composition, formulated as described above.

See, *e.g.*, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988. In one such technique, an antigenic portion of *pknB*, *pknG*, *pknH*, or *pknJ* polypeptide is initially injected into any of a wide variety of mammals (*e.g.*, mice, rats, rabbits, sheep or goats). Alternatively, in order to generate antibodies to relatively short peptide portions of *pknB*, *pknG*, *pknH*, or *pknJ*, a superior immune response may be elicited if the polypeptide is joined to an immunogenic carrier, such as ovalbumin, BSA, KLH, pre-S HBsAg, other viral or eukaryotic proteins, and the like. The peptide-conjugate is injected into the animal host, preferably according to a predetermined schedule incorporating one or more booster immunizations, and the animals are bled periodically. Polyclonal antibodies specific for the polypeptide may then be purified from such anti-sera by, for example, affinity chromatography using the polypeptide coupled to a suitable solid support.

Alternatively, for monoclonal antibodies, hybridomas may be formed by isolating the stimulated immune cells, such as those from the spleen of the inoculated animal. These cells are then fused to immortalized cells, such as myeloma cells or transformed cells, which are capable of replicating indefinitely in cell culture, thereby producing an immortal, immunoglobulin-secreting cell line. The immortal cell line utilized is preferably selected to be deficient in enzymes necessary for the utilization of certain nutrients. Many such cell lines (such as myelomas) are known to those skilled in the art, and include, for example: thymidine kinase (TK) or hypoxanthine-guanine phosphoriboxyl transferase (HGPRT). These deficiencies allow selection for fused cells according to their ability to grow on, for example, hypoxanthine aminopterinthymidine medium (HAT).

Preferably, the immortal fusion partners utilized are derived from a line that does not secrete immunoglobulin. The resulting fused cells, or hybridomas, are cultured under conditions that allow for the survival of fused, but not unfused, cells and the resulting colonies screened for the production of the desired monoclonal antibodies. Colonies producing such antibodies are cloned,

expanded, and grown to produce large quantities of antibody, see Kohler and Milstein, Nature (1975) 256:495 .

Large quantities of monoclonal antibodies from the secreting hybridomas may be produced by injecting the clones into the peritoneal cavity of mice and harvesting the ascites fluid therefrom. The mice, preferably primed with pristine, or some other tumor-promoter, and immunosuppressed chemically or by irradiation, may be any of various suitable strains known to those in the art. The ascites fluid is harvested from the mice and the monoclonal antibody purified therefrom, for example, by CM Sepharose™ column chromatography or other chromatographic means. Alternatively, the hybridomas may be cultured *in vitro* or as suspension cultures. Batch, continuous culture, or other suitable culture processes may be utilized. Monoclonal antibodies are then recovered from the culture medium or supernatant. It is preferred that such antibodies by humanized or chimerized according to one of the procedures outlined below.

In addition, the antibodies or antigen binding fragments may be produced by genetic engineering. In this technique, as with the standard hybridoma procedure, antibody-producing cells are sensitized to the desired antigen or immunogen. The messenger RNA isolated from the immune spleen cells or hybridomas is used as a template to make cDNA using PCR amplification. A library of vectors, each containing one heavy chain gene and one light chain gene retaining the initial antigen specificity, is produced by insertion of appropriate sections of the amplified immunoglobulin cDNA into the expression vectors. A combinatorial library is constructed by combining the heavy chain gene library with the light chain gene library. This results in a library of clones which co-express a heavy and light chain (resembling the Fab fragment or antigen binding fragment of an antibody molecule). The vectors that carry these genes are co-transfected into a host (*e.g.* bacteria, insect cells, mammalian cells, or other suitable protein production host cell.). When antibody gene synthesis is induced in the transfected host, the heavy and light chain proteins self-assemble

to produce active antibodies that can be detected by screening with the antigen or immunogen.

Preferably, recombinant antibodies are produced in a recombinant protein production system which correctly glycosylates and processes the immunoglobulin chains, such as insect or mammalian cells, as is known in the art.

Anti-STPK antibodies or moieties thereof find for use assaying, therapeutic and imaging purposes. Such antibodies, which may be selected as described above, may be utilized without further modification to include a cytotoxic or imaging moiety, or may be modified by conjugation to include such cytotoxic or imaging agents.

As used herein, "cytotoxic moiety" (C) simply means a moiety that inhibits cell growth or promotes cell death when proximate to or absorbed by the cell. Suitable cytotoxic moieties in this regard include radioactive isotopes (radionuclides), chemotoxic agents such as differentiation inducers and small chemotoxic drugs, toxin proteins, and derivatives thereof. As utilized herein, "imaging moiety" (I) means a moiety which can be utilized to increase contrast between infected and healthy tissue in a visualization technique (*e.g.*, radiography, positron-emission tomography, magnetic resonance imaging, direct or indirect visual inspection.) Thus, suitable imaging moieties include radiography moieties (*e.g.* heavy metals and radiation emitting moieties), positron emitting moieties, magnetic resonance contrast moieties, and optically visible moieties (*e.g.*, fluorescent or visible-spectrum dyes, visible particles, *etc.*).

Optically visible moieties for use as imaging moieties include fluorescent dyes, or visible-spectrum dyes, visible particles, and other visible labeling moieties. Fluorescent dyes such as fluorescein, coumarin, rhodamine, bodipy Texas red, and cyanine dyes, are useful when sufficient excitation energy can be provided to the site to be inspected visually. Endoscopic visualization procedures may be

more compatible with the use of such labels. Acceptable dyes include FDA-approved food dyes and colors, which are non-toxic, although pharmaceutially acceptable dyes which have been approved for internal administration are preferred. In preferred embodiments, such dyes are encapsulated in carrier moieties, which are in turn conjugated to the antibody. Alternatively, visible particles, such as colloidal gold particles or latex particles, may be coupled to the antibody moiety via a suitable chemical linker.

For administration, the antibody-therapeutic or antibody-imaging agent will generally be mixed, prior to administration, with a non-toxic, pharmaceutically acceptable carrier substance. Usually, this will be an aqueous solution, such as normal saline or phosphate-buffered saline (PBS), Ringer's solution, lactate-Ringer's solution, or any isotonic physiologically acceptable solution for administration by the chosen means. Preferably, the solution is sterile and pyrogen-free, and is manufactured and packaged under current Good Manufacturing Processes (GMP's), as approved by the FDA. The clinician of ordinary skill is familiar with appropriate ranges for pH, tonicity, and additives or preservatives when formulating pharmaceutical compositions for administration by intravascular injection, intrathecal injection, or by other routes. In addition to additives for adjusting pH or tonicity, the antibody-therapeutics and antibody-imaging agents may be stabilized against aggregation and polymerization with amino acids and non-ionic detergents, polysorbate, and polyethylene glycol. Optionally, additional stabilizers may include various physiologically-acceptable carbohydrates and salts. Also, polyvinylpyrrolidone may be added in addition to the amino acid. Suitable therapeutic immunoglobulin solutions which are stabilized for storage and administration to humans are described in U.S. Patent No. 5,945,098. Other agents, such as human serum albumin (HSA), may be added to the therapeutic or imaging composition to stabilize the antibody conjugates.

Antibodies coupled to cytotoxic moieties will recognize their targets within the body, where the cytotoxic moiety is brought in contact to or in close proximity to

the site of bacterial infection, whereupon the cytotoxic moiety interferes with the *Mycobacteria* and reduces its growth, prevents cell division, or otherwise renders it benign. Antibodies coupled to imaging moieties will recognize their targets within the body, whereupon their targets can be visualized using suitable methods described above, as is appropriate for the imaging moiety used.

For convenience, the term "antibody" or "antibody moiety" will be used throughout to generally refer to molecules which specifically bind to a *pknB*, *pknG*, *pknH*, or *pknJ* epitope.

Antisense molecules can be used to down-regulate gene expression in cells. The antisense reagent may be antisense oligonucleotides (ODN), particularly synthetic ODN having chemical modifications from native nucleic acids, or nucleic acid constructs that express such antisense molecules as RNA. The antisense sequence is complementary to the mRNA of the targeted gene, and inhibits expression of the targeted gene products. It has been found that short oligonucleotides, of from 7 to 8 bases in length, can be strong and selective inhibitors of gene expression (see Wagner *et al.*, Nature Biotechnology (1996) 14:840-844).

Antisense oligonucleotides may be chemically synthesized by methods known in the art (see Wagner *et al.* (1993) *supra*. and Milligan *et al.*, *supra*.) Preferred oligonucleotides are chemically modified from the native phosphodiester structure, in order to increase their intracellular stability and binding affinity. A number of such modifications have been described in the literature, which alter the chemistry of the backbone, sugars or heterocyclic bases.

Tissue Samples

For the purposes of detecting or assaying TB or STPKs in animals including man, samples such as infected tissue, *e.g.* washes, swabs, scrapings, excisions, biopsies, and blood samples, or cultures of TB grown up from such samples, *etc.*, may be used.

Candidate Agents

Samples of interest include, but are not limited to, chemical libraries, isolated chemicals, ODN as described above, and antibodies.

Assay Methods

*Mycobacteria*l serine/threonine protein kinases (STPK) can be used in drug screening methods to identify candidate therapeutic agents and other therapeutic targets.

In a typical assay, a sample, *e.g.* isolated chemical compound or compound library, *etc.* is assayed for the presence of STPK specific sequences by combining the sample with a specific binding member, and detecting directly or indirectly the presence of the complex formed between the two members. The term "specific binding member" as used herein refers to a member of a specific binding pair, *i.e.* two molecules where one of the molecules through chemical or physical means specifically binds to the other molecule. The complementary members of a specific binding pair are sometimes referred to as a ligand and receptor.

Binding pairs of interest include STPK and substrate pairs; phosphorylated or unphosphoryled kinases and antibodies specific therfore, and the like. The specific binding pairs may include analogs, derivatives and fragments of the original specific binding member. For example, an antibody directed to a protein antigen may also recognize peptide fragments, chemically synthesized peptidomimetics, labeled protein, derivatized protein, *etc.* so long as an epitope is present.

Substrates and methods for kinase assays are known in the art and are disclosed, for example, in published PCT application WO0138877. ATP and myelin basic protein are examples of such substrates. MBP and other peptides that are capable of being phosphorylated on serine and threonine residues are examples of such substrates for STPKs, but are not limiting.

Modulation of Enzyme Activity *in vivo* or *in vitro*

Agents that modulate activity of STPKs provide a point of intervention in an important signaling pathway. Agents that are useful in reducing this activity include agents that directly modulate expression, *e.g.* expression vectors, antisense specific for the targeted kinase; and agents that act on the protein, *e.g.* specific antibodies and analogs thereof, and small organic molecules that block catalytic activity, *etc.*

Therapeutic agents validated by the assays of the invention may be administered to inhibit STPK activity either *in vitro* or *ex vivo*. Therapeutic agents include small molecules, antibody or antibody moieties, peptides, and antisense, *etc.*

Antisense sequences may be administered to inhibit expression. Pseudo-substrate inhibitors, for example, a peptide that mimics a substrate for the kinase may be used to inhibit activity. Other inhibitors are identified by screening for biological activity in a functional assay, *e.g. in vitro* or *in vivo* kinase activity.

Detection of the STPKs may be used to establish efficacy or mode of action of various candidate drugs, and may utilize staining of infected cells or histological sections, performed in accordance with conventional methods. The above-described antibodies or other specific binding members of interest are added to, say, a tissue sample (see above), and incubated for a period of time sufficient to allow binding to the epitope, usually at least about 10 minutes. The antibody may be labeled with radioisotopes, enzymes, fluorescers, chemiluminescers, or other labels for direct detection. Alternatively, a second stage antibody or reagent is used to amplify the signal. Such reagents are well known in the art. For example, the primary antibody may be conjugated to biotin, with horseradish peroxidase-conjugated avidin added as a second stage reagent. Final detection uses a substrate that undergoes a color change in the presence of the peroxidase. The absence or presence of antibody binding may be determined by various methods, including flow cytometry of dissociated cells, microscopy, radiography, scintillation counting, *etc.*

Various insoluble supports for the assays may any compositions to which the antibodies or polypeptides can be bound, which is readily separated from soluble material, and which is otherwise compatible with the overall method. The surface of such supports may be solid or porous and of any convenient shape. Examples of suitable insoluble supports to which the receptor is bound include beads, *e.g.* magnetic beads, membranes and microtiter plates. These are typically made of glass, plastic (*e.g.* polystyrene), polysaccharides, nylon or nitrocellulose. Microtiter plates are especially convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples.

Sample treated lysates or test compounds are then added to separately assayable supports (for example, separate wells of a microtiter plate) containing antibodies. Preferably, a series of standards, containing known concentrations of the test protein is assayed in parallel with the samples or aliquots thereof to serve as controls. Preferably, each sample and standard will be added to multiple wells so that mean values can be obtained for each. The incubation time should be sufficient for binding, generally, from about 0.1 to 3 hr is sufficient. After incubation, the insoluble support is generally washed of non-bound components. Generally, a dilute non-ionic detergent medium at an appropriate pH, generally 7-8, is used as a wash medium. From one to six washes may be employed, with sufficient volume to thoroughly wash non-specifically bound proteins present in the sample.

After washing, a solution containing a second antibody or labeled substrate is applied. These will bind to the antibody or polypeptide of the invention with sufficient specificity such that it can be distinguished from other components present and may be labeled to facilitate direct, or indirect quantification of binding. Examples of labels that permit direct measurement of second receptor binding include radiolabels, such as ^{3}H or ^{125}I, fluorescers, dyes, beads, chemilumninescers, colloidal particles, and the like. Examples of labels that permit indirect measurement of binding include enzymes where the substrate may provide for a colored or fluorescent product. In a preferred embodiment, the

antibodies are labeled with a covalently bound enzyme capable of providing a detectable product signal after addition of suitable substrate. Examples of suitable enzymes for use in conjugates include horseradish peroxidase, alkaline kinase, malate dehydrogenase and the like. Where not commercially available, such antibody-enzyme conjugates are readily produced by techniques known to those skilled in the art. The incubation time should be sufficient for the labeled ligand to bind available molecules. Generally, from about 0.1 to 3 hr is sufficient, usually 1 hr sufficing.

After the second binding step, the insoluble support is again washed free of non-specifically bound material, leaving the specific complex formed between the target protein and the specific binding member. The signal produced by the bound conjugate is detected by conventional means. Where an enzyme conjugate is used, an appropriate enzyme substrate is provided so a detectable product is formed.

Other immunoassays are known in the art and may find use as diagnostics. Ouchterlony plates provide a simple determination of antibody binding. Western blots may be performed on protein gels or protein spots on filters, using a detection system specific for one of the proteins of the invention as desired, conveniently using a labeling method as described for the sandwich assay.

In some embodiments, the methods are adapted for use *in vivo*, *e.g.*, to locate or identify sites where *M. tuberculosis* infection is present. In these embodiments, a detectably-labeled moiety, *e.g.*, an antibody, which is specific for *pknB*, *pknG*, *pknH*, or *pknJ* is administered to an individual (*e.g.*, by injection), and labeled cells are located using standard imaging techniques, including, but not limited to, magnetic resonance imaging, computed tomography scanning, and the like. In this manner, bacterial cells are differentially labeled.

The detection methods can be provided as part of a kit. Thus, the invention further provides kits for detecting the presence of an STPK mRNA, and/or a

polypeptide encoded thereby, in a biological sample. Procedures using these kits can be performed by clinical laboratories, experimental laboratories, medical practitioners, or private individuals. The kits of the invention for detecting a polypeptide comprise a moiety that specifically binds the polypeptide, which may be a specific antibody. The kits of the invention for detecting a nucleic acid comprise a moiety that specifically hybridizes to such a nucleic acid. The kit may optionally provide additional components that are useful in the procedure, including, but not limited to, buffers, developing reagents, labels, reacting surfaces, means for detection, control samples, standards, instructions, and interpretive information.

Screening for Therapeutic Agents

Compound screening may be performed using an *in vitro* model, a genetically altered cell or animal, or purified STPK protein. One can identify ligands or substrates that bind to, modulate or mimic the action of the encoded polypeptide. Areas of investigation include the development of treatments for *Mycobacterial* infection.

The polypeptides include native *PknB*, *PknG*, *PknH*, or *PknJ*, as well as nonnative polypeptides that, by virtue of the degeneracy of the genetic code or mutations in the bacterial genome, are not identical in sequence to the disclosed native polypeptides. Variant polypeptides can include amino acid (aa) substitutions, additions or deletions. The aa substitutions can be conservative amino acid substitutions or substitutions to eliminate non-essential amino acids, such as to alter a glycosylation site, a phosphorylation site or an acetylation site, or to minimize misfolding by substitution or deletion of one or more cysteine residues that are not necessary for function. Variants can be designed so as to retain or have enhanced stability or other characteristics for drug screening. Variants also include fragments of the polypeptides disclosed herein, particularly biologically active fragments and/or fragments corresponding to functional domains. Fragments of interest will typically be at least about 10 aa to at least about 15 aa in length, usually at least about 50 aa in length, and can be as long

as 300 aa in length or longer, but will usually not exceed about 500 aa in length, where the fragment will have a contiguous stretch of amino acids that is identical to native *pknB*, *pknG*, *pknH*, or *pknJ*, or a homolog thereof.

Compound screening identifies agents that modulate function of *pknB*, *pknG*, *pknH*, or *pknJ*. Agents that mimic the function of these kinases are predicted to activate the process bacterial infection. Conversely, agents that inhibit function may inhibit infection or progression of disease. Of particular interest are screening assays for agents that have a low toxicity for human cells. A wide variety of assays may be used for this purpose, including labeled *in vitro* protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, and the like. Knowledge of the 3-dimensional structure of the encoded protein, derived from crystallization of purified recombinant protein, could lead to the rational design of small drugs that specifically inhibit activity. These drugs may be directed at specific domains, *e.g.* the kinase catalytic domain, the regulatory domain, the auto-inhibitory domain, *etc.*

Kinase inhibitors of use in the invention include isoquinolinesulfonyl compounds or salts thereof which occupy the ATP binding site of the enzyme. Examples of this compound family are known in the art and include: W-7 (Hidaka *et al.*, 1978); ML-9 (Saitoh *et al.*, 1987); CKI-7 (Chijiwa *et al.*, 1989); H-9 (Hidaka *et al.*, 1984); H-8 (Hidaka *et al.*, 1984); H-7 (Hidaka *et al.*, 1984); HA1077 (Takayasu *et al.*, 1986); H-89 (Chijiwa *et al.*, 1990); CKA-1306 (Sakaguchi *et al.*, 1998); KN-62 (Tokumitsu *et al.*, 1990) (Ono-Saito *et al.*, 1999).

Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically one of these concentrations serves as a negative control, *i.e.* at zero concentration or below the level of detection.

Candidate therapeutic agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having

a molecular weight of more than 50 and less than about 2,500 Daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amplification, *etc.* to produce structural analogs.

Where the screening assay is a binding assay, one or more of the molecules may be joined to a label, where the label can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemiluminescers, enzymes, specific binding molecules, particles, *e.g.* magnetic particles, and the like. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin, *etc.* For the specific binding members, the complementary member would normally be labeled with a molecule that provides for detection, in accordance with known procedures.

A variety of other reagents may be included in the screening assay. These include reagents like salts, neutral proteins, *e.g.* albumin, detergents, *etc.* that are used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, *etc.* may be used. The mixture of components are added in any order that provides for the requisite binding. Incubations are performed at any suitable temperature, typically between 4 and 40° C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening. Typically between 0.1 and 1 hours will be sufficient.

The compounds having the desired pharmacological activity may be administered in a physiologically acceptable carrier to a host for treatment of *Mycobacterial* infection such as tuberculosis, *etc.* The inhibitory agents may be administered in a variety of ways, orally, by nasal or pulmonary inhalation, topically, parenterally *e.g.* subcutaneously, or intraperitoneally, intravascularly, *etc.* Depending upon the manner of introduction, the compounds may be formulated in a variety of ways. The concentration of therapeutically active compound in the formulation may vary from about 0.1-10 wt %.

Formulations of Agents

The compounds of this invention can be incorporated into a variety of formulations for therapeutic administration. Particularly, agents that modulate *pknB*, *pknG*, *pknH*, or *pknJ* activity are formulated for administration to patients for the treatment of cells where the infection is present. More particularly, the compounds of the present invention can be formulated into pharmaceutical compositions by combination with appropriate, pharmaceutically acceptable carriers or diluents, and may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants, gels, microspheres, and aerosols. As such, administration of the compounds can be achieved in various ways, including oral, buccal, rectal, parenteral, intraperitoneal, intradermal, transdermal,

intra-tracheal, *etc.*, administration. The agent may be systemic after administration or may be localized by the use of an implant that acts to retain the active dose at the site of implantation.

In pharmaceutical dosage forms, the compounds may be administered in the form of their pharmaceutically acceptable salts, or they may also be used alone or in appropriate association, as well as in combination with other pharmaceutically active compounds. The following methods and excipients are merely exemplary and are in no way limiting.

For oral preparations, the compounds can be used alone or in combination with appropriate additives to make tablets, powders, granules or capsules, for example, with conventional additives, such as lactose, mannitol, corn starch or potato starch; with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch or gelatins; with disintegrators, such as corn starch, potato starch or sodium carboxymethylcellulose; with lubricants, such as talc or magnesium stearate; and if desired, with diluents, buffering agents, moistening agents, preservatives and flavoring agents.

The compounds can be formulated into preparations for injections by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

The compounds can be utilized in aerosol formulation to be administered via inhalation. The compounds of the present invention can be formulated into pressurized acceptable propellants such as dichlorodifluoromethane, propane, nitrogen and the like.

Furthermore, the compounds can be made into suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases. The compounds of the present invention can be administered rectally via a suppository. The suppository can include vehicles such as cocoa butter, carbowaxes and polyethylene glycols, which melt at body temperature, yet are solidified at room temperature.

Unit dosage forms for oral or rectal administration such as syrups, elixirs, and suspensions may be provided wherein each dosage unit, for example, teaspoonful, tablespoonful, tablet or suppository, contains a predetermined amount of the composition containing one or more compounds of the present invention. Similarly, unit dosage forms for injection may comprise the compound of the present invention in a composition as a solution in sterile water, normal saline or another pharmaceutically acceptable carrier.

Implants for sustained release formulations are well-known in the art. Implants are formulated as microspheres, slabs, *etc.* with biodegradable or non-biodegradable polymers. For example, polymers of lactic acid and/or glycolic acid form an erodible polymer that is well-tolerated by the host. The implant is placed in proximity to the site of disease, so that the local concentration of active agent is increased relative to the rest of the body.

The term "unit dosage form," as used herein, refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of compounds of the present invention calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier or vehicle. The specifications for the novel unit dosage forms of the present invention depend on the particular compound employed and the effect to be achieved, and the pharmacodynamics associated with each compound in the host.

The pharmaceutically acceptable excipients, such as vehicles, adjuvants, carriers or diluents, are readily available to the public. Moreover, pharmaceutically acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are readily available to the public.

Typical dosages for systemic administration range from 0.1 µg to 100 milligrams per kg weight of subject per administration. A typical dosage may be one tablet taken from two to six times daily, or one time-release capsule or tablet taken once a day and containing a proportionally higher content of active ingredient. The time-release effect may be obtained by capsule materials that dissolve at different pH values, by capsules that release slowly by osmotic pressure, or by any other known means of controlled release.

Those of skill will readily appreciate that dose levels can vary as a function of the specific compound, the severity of the symptoms and the susceptibility of the subject to side effects. Some of the specific compounds are more potent than others. Preferred dosages for a given compound are readily determinable by those of skill in the art by a variety of means.

The use of liposomes as a delivery vehicle is one method of interest. The liposomes fuse with the cells of the target site and deliver the contents of the lumen intracellularly. The liposomes are maintained in contact with the cells for sufficient time for fusion, using various means to maintain contact, such as isolation, binding agents, and the like. In one aspect of the invention, liposomes are designed to be aerosolized for pulmonary administration. Liposomes may be prepared with purified proteins or peptides that mediate fusion of membranes, such as Sendai virus or influenza virus, *etc.* The lipids may be any useful combination of known liposome forming lipids, including cationic lipids, such as phosphatidylcholine. The remaining lipid will normally be neutral lipids, such as cholesterol, phosphatidyl serine, phosphatidyl glycerol, and the like.

While specific embodiments of the invention have been described and illustrated, such embodiments should be considered illustrative of the invention only and not as limiting the invention as construed in accordance with the accompanying claims.

The following examples are meant to help illustrate, but not limit, the present invention. The following bacterial strains, vectors, and culture conditions are used in the Examples.

M. bovis Bacille Calmette Guerin or BCG was obtained from ATCC.

M. smegmatis mc^2155 was obtained from Dr. William Jacobs at the Albert Einstein Medical School.

M. tuberculosis H37Rv NCTC 7416 was obtained from the National Collection of Type Cultures, London, U.K.

Escherichia coli DH5a (Clontech Laboratories, Inc., Palo Alto, Calif.) and *E. coli* BL21(DE3) (Novagen R & D) were used for maintenance of plasmids and expression of foreign proteins, respectively.

The plasmid pET-22b (Novagen) was used as an expression vector in *E. coli* BL21(DE3).

E. coli strains were cultured on Luria-Bertani (LB) agar or broth with or without selective antibiotics. *Mycobacterial* strains were cultured in Middlebrook™ 7H9 broth or 7H10 agar (Difco) supplemented with OADC (Difco), Tween 80, and glycerol.

EXAMPLE 1

Amplification and cloning of *pknB*, *pknG*, *pknH*, and *pknJ*

Genomic DNA of *M. tuberculosis* H37Rv was prepared as described previously (Av-Gay, Y. et al., 2000). The open reading frame Rv 0014c which codes for *PknB* was amplified using two primers containing NdeI and HindIII restriction sites, respectively. PCR was performed with Taq™ polymerase obtained from Gibco BRL by using 2 mM $MgCl_2$ and 5% DMSO. Annealing temperatures were 58 and 63°C. The PCR products were separated on a 1% agarose gel. The appropriate PCR product was ligated into the vector pCR2.1 of the TA cloning kit (Invitrogen) and transformed into *E. coli* DH5a or INVF9a by standard chemical transformation procedure. Clones containing the vector were selected on LB-plus-ampicillin (100 mg/ml) plates, and plasmid DNA was digested with restriction endonucleases NdeI and HindIII (Fermentas). Restriction enzyme-digested plasmids were isolated with a QIAquick™ gel extraction kit (Qiagen Ltd.). A corresponding digestion was also applied to plasmid pET-22b, and the two products were ligated together with T4 DNA ligase to obtain the plasmid pYA102.

cDNA preparation and RT-PCR

RNA was prepared in a P3-level laboratory as follows. Exponentially growing *M. tuberculosis* (H37Rv), J774.2 cells infected with *M. tuberculosis* or adherent alveolar macrophages from bronchoalveolar lavage (BAL) fluid, were harvested by centrifugation at 3,000rpmX 3g for 10 min. Pellets were resuspended in 2 ml of Tween-saline (0.8% [wt/vol] NaCl, 0.05% [vol/vol] Tween 80) followed by centrifugation at 17,000X3g for 1 min. The *Mycobacteria*l pellets were resuspended in 1 ml of guanidinium isothiocyanate buffer (5 M guanidinium isothiocyanate, 25 mM sodium acetate [pH 6.0], 1% N-lauryl sarcosine, 10 mM DTT), and approximately 1 ml of 0.1-mm-diameter zirconium beads was added. *Mycobacteria* were then subjected to disruption in a bead-beater device for 3 min. Nucleic acid was prepared from the upper aqueous lysate by a series of chloroform and phenol-chloroform-isoamyl alcohol extractions followed by ethanol precipitation. To remove DNA template, RNA was treated with RNase-

free DNase (RQ1™; Promega) twice at a concentration of 1 U/mg of RNA. DNase was removed by phenol-chloroform-isoamyl alcohol extraction, followed by extraction with chloroform and ethanol precipitation. RNA pellets were resuspended in diethylpyrocarbonate-treated water, and the absence of DNA contamination was confirmed by PCR with specific primers for *STPK*s. RNA concentrations were determined by measuring absorbance at 260 nm. Total RNA was heated at 94°C for 3 min before being cooled on ice for 5 min. RNA was reverse transcribed by adding PCR buffer, 1.5 mM MgCl2, 200 mM each of the four deoxynucleotide triphosphates, 5 nM random hexamers or specific downstream nucleotides, and 50 U of murine leukemia virus reverse transcriptase (Perkin-Elmer). RNA was reverse transcribed at 42°C for 3 h. Murine leukemia virus was inactivated by incubation at 99°C for 3 min. Amplified products were produced routinely with either 20 ng of genomic *M. tuberculosis* DNA, as a positive control, or 5 to 10 ng of cDNA which was added to 20 ml containing PCR buffer, 200 mM deoxynucleotide triphosphates, 4% dimethyl sulfoxide, 200 pmol of each primer, and 1 U of Taq polymerase (Fermentas). The primers for RT-PCR analysis were the same primers used for the gene cloning. The amplification program consisted of preamplification denaturation at 95°C for 3 min, followed by 35 cycles of 60s of denaturation at 94°C, 60 s of annealing at 58°C, and a 90 s extension at 72°C. Ten percent of the reaction product was run on a 1% agarose gel and visualized by staining with ethidium bromide.

Expression and purification of *Mycobacterial* STPK

Competent cells of *E. coli* BL21(DE3) were prepared according to the $CaCl_2$ method (Sambrook, J et al. 1989) and were transformed using heat shock for 2 min at 42°C with 100 ng of pYA102. The transformed *E. coli* cells were then plated onto LB agar supplemented with ampicillin (100 mg/ml). Single colonies were inoculated into 5 ml of LB broth also containing ampicillin (100 mg/ml). After overnight incubation at 37°C with shaking, the individual cultures were diluted 1:100 in the same medium and incubation was continued at 37°C with shaking. Isopropyl-b-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM when the optical density at 600 nm reached 0.6. Cultures were

centrifuged at 5,000 3 g for 15 min at room temperature, and pellets were lysed in B-Per™ (Pierce) bacterial protein extraction reagent. Proteins were separated by centrifugation (15,000X3 g, 4°C, 15 min) into soluble and insoluble fractions.

STPK inclusion bodies contained in the insoluble fractions were purified from *E. coli* membrane proteins by washing in a solution of 10% B-Per reagent and centrifugation (45,000X3 g, 4°C, 90 min). *STPK* was separated by sodium dodecyl sulfate–7.5% polyacrylamide electrophoresis (SDSPAGE) and stained with Coomassie blue or transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The N-terminal amino acid sequence was verified after electrophoresis of samples in SDS-PAGE gels and electroblotting onto PVDF membranes. Edman degradation was performed, and the sequence of the first 10 amino acids from the NH_2 terminus was determined at the University of British Columbia Protein Sequencing Laboratory. In order to obtain soluble protein, *STPK* inclusion bodies were resuspended in phosphate-buffered saline (PBS) (pH 7.4) and slowly added drop-wise to a solution of 16 M urea and 2 M dithiothreitol (DTT) to make a final concentration of 8 M urea and 1 M DTT. Soluble *STPK* was then dialyzed using Spectra/Por™ 8000 cellulose membrane (VWR Scientific) against 200 volumes of 13 Tris-buffered saline (pH 7.4) at 4°C for 16 to 24 h. The sample was then centrifuged for 15,000X3 g, at 4°C for 15 min, and approximately 20 mg of protein was loaded onto a 50-ml Macro-Prep™ SE agarose size-exclusion column (Bio-Rad), which was used as a desalting column. Proteins were eluted over time at 4°C with a size-exclusion buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM DTT, and 0.01 mM EDTA. The purity of the *STPK* was tested by subjecting samples to SDSPAGE followed by Coomassie blue staining. SDS-PAGE gels were prepared by the method of Laemmli et al. (1970). The gels were stained with Coomassie blue R-250 or silver stain. Protein concentrations were determined by the Bradford protein assay reagent (Bio-Rad).

EXAMPLE 2

Production of antibodies to *STPK*

The gel electrophoresis bands corresponding to the various *STPK*s were excised from an SDS–7.5% PAGE gel and homogenized in PBS. Homogenized gels were mixed with Titremax™ adjuvant (1:1 [vol/vol]) and were injected subcutaneously into 8-week-old BALB/c mice. Two weeks after the immunization, the animals were bled and sera were prepared. Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) antibodies and enhanced chemiluminescence reagents were used to detect antibodies bound in *in vitro* kinase assay.

STPK autophosphorylation and myelin basic protein (MBP) phosphorylation were determined by an *in vitro* kinase assay. Twenty µm samples of *STPK* protein were added to 15 ml of kinase buffer [20 mM PIPES [piperazine-N,N9-bis(2-ethanesulfonic acid); pH 7.2] 10 mM $MgCl_2$] with or without 50 mg of MBP (Sigma), and the reaction was started by addition of 1 mCi of [g-^{32}P]ATP (Mandel Scientific). Incubation was performed at room temperature. One-third of the incubation mixture was then loaded onto P81 phosphocellulose filter paper (Baxter) for incorporation measurements, and to the remaining Laemmli sample buffer was added to stop the reaction. The latter mixture was boiled for 5 min and resolved by SDS-PAGE. The gels were electroblotted onto nitrocellulose or PVDF membranes (Bio-Rad) and then exposed to Kodak X-Omat/AR film. Radioisotope levels in filter paper assays were determined by scintillation counting (Beckman LS 1800).

Phosphoamino acid analysis, Western blotting, and cross-reactivity with antieukaryotic STPKs

Autophosphorylated *STPK*s was excised from PVDF membranes and subjected to acid hydrolysis as described by Kamps and Sefton (1989). Samples were spotted onto a cellulose thin-layer chromatography plate (Eastman, Rochester, N.Y.) and subjected to two-dimensional liquid thin-layer chromatography. Control

phosphotyrosine, phosphothreonine, and phosphoserine amino acids were stained with ninhydrin, and radiolabelled amino acids were visualized via autoradiography. Phosphoamino acid analysis was also performed by loading *STPK*s on SDS-PAGE gels followed by Western blot analysis with antiphosphoserine and antiphosphothreonine monoclonal antibodies (Sigma) as described in the manufacturer's instructions. Soluble *STPK* was electrophoresed on an SDS–7.5% PAGE gel and transferred to nitrocellulose by semidry electroblotting (Ancos). Blots were blocked with 4% skim milk (Difco, Detroit, Mich.) in PBS (pH 7.5) overnight at 4°C on a shaker. Blots were washed with pBS–0.1% Tween 20 and incubated for 2 h in PBS–Tween (pH 7.5) with either mouse monoclonal antiphosphoserine (1/500) (Sigma), mouse monoclonal antiphosphothreonine (1/100) (Sigma), rabbit antiphospho Creb Ser-133 (1/10,000) (New England Biolabs), rabbit antiphospho p38 Tyr-182 (1/10,000) (New England Biolabs), rabbit antiphospho mitogen-activated protein kinase kinase 3 and 6 (MKK3/6) Ser 189-202 (1/10,000), or rabbit anti-ERK1 (1/10,000) (Kinetek Pharmaceuticals, Vancouver, British Columbia, Canada). Blots were washed for 45 min in PBS–Tween and incubated with 1/20,000 of either goat anti-rabbit IgG (heavy plus light chain) or goat anti-mouse IgG (heavy plus light chain) HRP-conjugated antibody (Bio-Rad). Blots were incubated in Super Signal reagent (Pierce, Rockford, Ill.) and exposed by using Kodak X-Omat/AR™ film.

EXAMPLE 3

Screening for kinase inhibitors against *Mycobacterial STPK*

PI3-kinase inhibitor wortmannin (Calbiochem, La Jolla, CA), isoquinolinesulfonyl H7 (ICN, Costa Mesa, CA), the alkyloid-like kinase inhibitor K252a from Nocardiopsis (Biomol, Plymouth Meeting, PA) and the protein serine/threonine phosphatase inhibitor okadaic acid (ICN, Costa Mesa, CA) were chosen as candidate drugs for validating the model. Due to the presence of the hydrophobic layers of mycolic acid layer and cell membrane, higher doses were used than usual for eukaryotic models.

Bacterial growth was measured by the microplate alamarBlue™ assay (Accumed Inernational, Westlake, OH). BCG was grown in 7H9 media at 37°C as described above. Bacteria were diluted to $1X10^6$ CFUs in 7H9 media (Collins, LA et al. 1997) and were treated with varying doses of kinase inhibitors. Measurement of optical densities were done as previously described by Yajko DM et al. in 1995. Media only controls, alamarBlue™ in media controls, BCG growth controls, and drug treatments were utilized to calculate the percent reduction of the cultures, which is directly related to *Mycobacteria*l growth.

The measures of viability were expressed as %viability or log (% of viability) compared to the initial bacilli input. By this standardization, results from multiple experiments over time could be compared.

Relative growth (%) = treated CFU X 100% X (initial dilution factor X100 stock culture)$^{-1}$

Kinase assay

Kinase assays were done at room temperature with the addition of dH_20 or increasing concentrations of drug diluted in dH_20 at both zero and 15 minute time points. Reactions were stopped by the addition of Laemmli's SDS-PAGE sample loading buffer. Proteins were separated by 12%SDS-PAGE and *PknB*-mediated MBP phosphorylation was detected by autoradiography.

The inhibitory effect of drug on *STPK*-mediated MBP phosphorylation was evaluated by densitometry of the autoradiography. Relative densities of MBP phosphorylation bands were measured by densitometry (Bio-Rad Quantity One™). At the highest concentration tested, wortmannin (0.5 μM), K252a (4 mM), and okadaic acid (6 μM) failed to inhibit the growth of BCG. At the same time, H7 limited the growth of BCG in liquid culture.

A dose of 20 μg ml^{-1} (3 mM) of H7 decreased the growth of BCG cultures, as measured by oxidative phosphorylation, to a level that was 60% of untreated

culture. This decrease in bacterial growth is roughly equivalent to that obtained by 20μg ml^{-1} (0.035 mM) of the bactericidal antibiotic hygromycin.

To exclude the possibility that the effect of H7 was due to a non-specific toxic effect, the effect of H7 on *E. coli* HB101 was examined. *E. coli* lacks any STPKs and indeed its growth was not inhibited by concentrations of up to 3 mM H7, over 24 h growth.

EXAMPLE 4

H7 limits *Mycobacterial* growth over a 48 h period as measured by viable counts

The question of whether H7 directly inhibits bacterial growth of *M. smegmatis* mc^2155 was investigated. *M. smegmatis* was chosen because of its relatively rapid growth rate and because its genome contains STPK homologs. A dose response to H7 was evaluated after 24 h of growth or approximately 4-7 generations. H7 had a dose-dependent inhibitory effect on *M. smegmatis* growth. A maximal dose of 3 mM H7 induced a one-log decrease in *M. smegmatis* CFUs when compared to solvent only groups. In the positive control group, 0.03 mM hygromycin induced a decrease in *M. smegmatis* viable count.

To identify the optimal time for H7 inhibition of *M. smegmatis* growth, bacteria were treated over 0-48 h. In three separate experiments a limiting dose of 3mM H7 induced a two-log decrease in *M. smegmatis* growth when compared to solvent treatment controls within 24 h. The positive control, a dose of 0.03mM hygromycin, cleared plates within a within a 24 h period. This series of time course experiments provided evidence that the inhibitory effect of H7 is evident over a period of several *Mycobacterial* generations.

H7-limited BCG growth correlates to a decrease in viable counts over a 10-day period. To determine if the initial decrease in BCG culture oxidative phosphorylation, correlates to a decrease in viability in liquid media, a time course assay over a 10-day period using the limiting doses of drugs optimized in

the initial screening experiments was performed. Over three separate experiments, the inhibitor H7 (3mM) induced a 50% decrease in the relative percentage growth (mean +/- S.E.M.) of BCG when compared to non-treatment groups on day 10. The positive control, 0.03 mM hygromycin, effectively killed BCG throughout the same time period. This confirms that the H7-induced growth inhibition seen in the alamarBlue™ example was due to the limitation of BCG viability and not due to interference with some other metabolic process.

EXAMPLE 5

H7 inhibits *pknB* activity *in vitro*

In addition to being a kinase inhibitor, H7 has also been shown to inhibit other molecular targets (such as RNA polymerase II) which are involved in growth and development. Since H7 has a variable effect on eukaryotic kinases, it was unclear whether *Mycobacteria*l STPKs themselves are inhibited by this small molecule. To determine if H7 targets *Mycobacteria*l STPKs, we performed *in vitro* kinase assays using the *M. tuberculosis* kinase *PknB* (Av-Gay, Y et al., 1999). A dose of 6 µM H7 was sufficient to inhibit *PknB*-mediated phosphorylation of artificial substrate MBP by 81% when compared to solvent alone. This difference between 0 µM and 6 µM H7 is significant as judged by Friedman's non-parametric analysis of variation (n=5, Friedman's Statistic 9.960, four groups, P = 0.0087 and Dunn's multiple comparison test n=5, P60.05). These data shows that H7 targets a *Mycobacteria*l STPKs.

EXAMPLE 6

Validation of *pknG* and *pknH* as Drug Targets

Construction of Δ*pknG*::hyg and Δ*pknH*::hyg mutants of *M. tuberculosis*.

Using *lacZ* selection (Av-Gay, Y. et al., 2000) as well as *sacB* counter selection (Pelicic *et al.*, 1996), Δ*pknG::hyg* and Δ*pknH::hyg* mutants in *M. tuberculosis* H37Rv were generated by two-step mutagenesis (Parish T., et al. 2000). The non-replicating plasmids *pknG*2.1 and *pknH*2.17 carrying the Hyg resistance

gene inserted into the deleted genes, were used to replace the wild type *pknG* and *pknH* by allelic exchange. After transformation, bacteria harboring *pknG*2.1 were plated onto media containing Hyg, Km and X-gal. Seven blue colonies arising from site-specific single crossover events were isolated, one of which was serially diluted and plated onto media containing Hyg, Suc and X-gal. Growth of white, Suc^R colonies resulting from the Suc selection step on media containing Hyg and X-gal was poor compared to the wild type (wt) control. Patching of Suc^R colonies onto Hyg, X-Gal and Km-containing media resulted in 15 white, Km^S colonies, which had presumably undergone a double crossover event. Of these, 12 were selected for genotypic analysis and were all shown to be knockout mutants. Restriction sites used to confirm the mutant genotype are shown for the wild type and mutant alleles in together with the Southern blot analysis. The 1202 base pair (bp) fragment observed in the wt and single crossover mutant is lost in the knockout mutants and replaced by the 5685 bp fragment resulting from the deletion of the *Bam*HI site present in wt *pknG* allele.

Bacteria harboring r *pknH*2.17 were also plated onto media containing Hyg, Km and X-gal. We were able to isolate 27 blue colonies arising from site-specific single crossover events, one of which was serially diluted and plated onto media containing Hyg, Suc and X-gal. Patching of white, Suc^R colonies onto Hyg, X-Gal and Km-containing media resulted in 17 white, Km^S colonies, which had presumably undergone a double crossover event. Of these, 12 were selected for genotypic analysis and were all shown to be knockout mutants. Restriction sites used to confirm the mutant genotype are shown for the wild type and mutant alleles in together with the Southern blot analysis. The 3723 bp fragment observed in the wt and single crossover mutant is lost in the knockout mutant and replaced by the 3154 bp fragment resulting from the Δ*pknH*::*hyg* allele.

Phenotypic analysis of the Δ*pknG*::Hyg^r and Δ*pknH*::Hyg^r strains in tuberculosis mouse model.

The Δ*pknG*::Hyg^r mutant strain was analysed for virulence in mouse model of tuberculosis. BALB/C mice were infected intravenously with $1X10^5$ CFU of three

strains of *M. tuberculosis* H37Rv; the parental *M. tuberculosis* H37Rv strain and two independent *pknG*::Hyg^r and *pknH*::Hyg^r strains. Data presented shows different levels of bacteria in Balb/C lung, spleen and liver, with mutant *pknG* and *pknG*-bearing bacteria lagging behind wt.

SCID mice were infected with wt and mutant strains to demonstrate virulence in an immunocompromised model. Five mice in each group were injected with the following amounts of bacteria, and with the following results:

Group	Innoculation	Life Span after Innoculation
M. tuberculosis H37Rv	1.84 X 10^5	31 days
pknG::Hyg^r	1.88 X 10^5	70+ days
pknH::Hyg^r	1.45 X 10^5	54 days
PknJ::Hyg^r	1.12 X 10^5	48 days

EXAMPLE 7

The *pknG* gene is the second gene in a potential 3-gene operon. In order to clone the promoter controlling *pknG* expression and analyze the transcriptional activity of *pknG*, we PCR amplified 2972 base pairs of DNA located upstream of the *pknG* gene that would encompass the first gene of the operon, as well as an additional 667 base pairs upstream of the first gene of the putative operon. This PCR fragment was then inserted into the *Mycobacterium-E. coli* shuttle vector pSC301 upstream of a promoterless green fluorescent protein (*gfp*) gene (Cowley, SC et al. 2001). The resulting plasmid was introduced into *M. smegmatis* in order to monitor transcriptional activity of *pknG* via spectrofluorometry. Transcription of *pknG* increases significantly in *M. smegmatis* cultures grown in bacteriological media upon entry into stationary phase. In comparison, transcriptional activity of another *M. tuberculosis*

serine/threonine kinase promoter, *pknH,* remained constant over the entire course of the growth curve.

We exposed cultures of *M. smegmatis* harboring the *pknG*-gfp fusion plasmid to a variety of conditions related to nutrient deprivation and long-term growth. Growth of *M. smegmatis* cultures in minimal media resulted in a 2 to 3-fold increase in *pknG* promoter activity. It was found that most stress conditions had either no effect or a negative effect on *pknG* promoter activity in *M. smegmatis* as compared to control cultures. Consistent with the observations described above, it was found that *pknG* promoter activity consistently increased by a factor of 2 to 4-fold in stationary phase (optical density of 1-2) as compared to *M. smegmatis* cultures in early exponential phase (optical density of 0.1-0.3). Thus, expression of the *pknG* gene is up-regulated upon entry into stationary phase.

EXAMPLE 8

PknG Coated Bead Assay

Latex beads coated with the mutant and wild type protein showed that the mutant *Mycobacteria PknG* can inhibit phagosome lysosome fusion by 30%. Fusion is a phenomenon typical of *M. tuberculosis* infection and these results show that *PknG* inhibiting therapies are useful in treating active infection.

Coating latex beads with soluble *PknG*

50 mL of 4 µM Latex beads were washed two times in 1 ml of ice cold 25mM 2-[N-Morpholino]ethanesulfonic acid (MES) buffer pH5.5, then centrifuged at full speed for 2 min between each wash. Beads were then mixed with (a) 100ug to 200ug of purified protein; (b) BSA. 50ul of 250mM MES buffer pH5.5 (10X buffer) was added to the mixture and the volume adjusted to 500ul. Beads were incubated O/N at RT, then washed 3 times with ice cold Hank's Balanced salt solution. (HBSS). The bead mixture was centrifuged then resuspended in 1ml of HBSS with 0.1% glycine pH7.2. They can be stored at 4°C. Successful coating of the beads was verified by SDS-PAGE.

Macrophages were infected with coated *PknG* beads

J774 cells were grown to 60 to 70% confluency on 6mm plates. Media was replaced with fresh J774 media containing 1% FCS (the regular media: DMEM, 1%penicllin-stretomycin, 1%glutamine, and 5% FCS).

FITC-Dextrane in RMPI was added to a final concentration of 1mg/ml, and the culture was incubated O/N at 37°C. Cells were washed 3X with 3 ml HBSS and then 3ml of J774 media was added. Another 4h of incubation at 37 °C was provided. Media was replaced with 1ml of fresh media and 25ul of coated beads mixture was added (volume added to the cells need to be determined under microscope) to 1ml of J774 media, then mixed well by vortexing.

The beads were added to the cells with fresh media and observed by microscope. The coated beads were allowed to settle on the cells at RT for 1/2 hr without vibration, then the cell mixture was incubated for another 2.5 hr at 37°C. Cells were washed with trypsin until the cells start to detach, then washed with HBSS. Cells were scraped off off the plate and resuspended in 500µl phagosome buffer (Phagosome buffer: Hepes 20mM, EGTA 0.5mM, sucrose 0.25M, NaN3 0.05%, Adjust pH to 7.4).

Cells were homogenized in 1ml volume 5-6 time, then the mixture diluted if required. Formaldehyde was added to a final concentration of 2.5% and FACS performed as per manufacturer's instructions (Becton Dickinson FACSCalibur).

EXAMPLE 9

Macrophage Assay Models

Murine macrophage infection

The macrophages as prepared are used in assaying the therapeutic agents. The variously infected macrophages are contacted with putative therapeutic agents in vitro and their survival is assayed and compared. A good therapeutic candidate will increase survival in comparison to nontreated macrophages.

For example, the mouse macrophage cell line J774.2 (American Type Culture Collection) is seeded and maintained in 100-mm-diameter tissue culture plates in RPMI 1640 medium (Gibco) containing 10 mM HEPES, 2 mML-glutamine, and 10% heat-inactivated fetal calf serum (FCS). For reverse transcription (RT)-PCR analysis, approximately 107 cells of *M. tuberculosis* H37Rv cultured in RPMI 1640 medium supplemented with 5% FCS were added to each J774.2 plate and incubated for 24 h. After 24 h, the cells were washed twice with warm RPMI 1640 medium containing 1% FCS. Total RNA was isolated after 24 and 72 h.

The macrophage model shows the same rate of infection for the wild type and mutant *Mycobacteria*, but the mutant does not survive as well as the parental strain.

Isolation of human alveolar macrophages

Human macrophages may be used as a humanized model in the same manner as the murine macrophages above described. In combination with potential therapeutic agents, the humanized model demonstrates efficacy.

For example, alveolar macrophages were obtained from bronchoalveolar lavage (BAL) fluid from resected human lungs. BAL fluid was filtered through sterile gauze and centrifuged at 450rpmX3 g at room temperature for 7 min. Erythrocytes were lysed by treatment with distilled water, and cells were washed twice with sterile PBS. Cells were resuspended in RPMI 1640 culture medium supplemented with 10% heat-inactivated FCS. Alveolar macrophages were plated in 60-mm-diameter tissue culture plates at 23 $X10^6$ cells/ml. Cells were allowed to adhere overnight at 37°C. Nonadherent cells were removed by gently rinsing the plates with warmed RPMI 1640 medium supplemented with 5% FCS. The protocol for using human biological samples was approved by the University of British Columbia Ethics Committee.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of skill in the art in light of the teachings of this invention that changes and modification may be made thereto without departing from the spirit or scope of the appended claims. All patents, patent applications and publications referred to herein are hereby incorporated by reference.

What is claimed is:

1. A method of screening for a modulator of PknG activity comprising:

i) contacting a sample containing PknG with a candidate agent;

ii) determining PknG activity in the sample of (i) subsequent to the contacting;

iii) comparing the activity determined in (ii) to PknG activity of a sample of (i) without the candidate agent present;

wherein, a difference in PknG activity in samples with and without the candidate agent present, is indicative that the candidate agent modulates PknG activity.

2. An assay for detecting modulation of PknG activity, comprising:

i) determining a first PknG activity level in a sample of PknG;

ii) determining a second PknG activity level of a sample of (i) further comprising a candidate agent;

iii) comparing the first and second levels;

wherein, difference in the first and second levels relates to an ability of the candidate agent to modulate PknG activity.

3. The method of claim 1 or 2 wherein samples comprising PknG further comprise a PknG substrate.

4. The method of claim 3 wherein the substrate is MPB.

5. The method of claim 3 or 4, wherein activity of PknG is determined by measuring phosphorylation of the substrate.

6. The method of claim 3 or 4, wherein activity of PknG is determined by measuring binding of the substrate to PknG.

7. The method of claim 1 or 2, wherein activity of PknG is determined by measuring binding of ATP to PknG.

8. The method of any one of claims 1-7, wherein a decrease in PknG activity in the presence of a candidate agent is indicative that the agent will decrease mycobacterial growth or survival.

9. The method of any one of claims 1-8, wherein the PknG is a *M. tuberculosis* PknG.

10. The method of any one of claims 1-9, wherein PknG is present on a solid support.

11. The method of any one of claims 1-10, wherein a PknG substrate is present on a solid support.

12. The method of any one of claims 1-11, wherein a sample without a candidate agent further comprises a PknG inhibitor.

13. The method of claim 12, wherein the inhibitor is an isoquinolinesulfonyl compound or salt thereof.

14. A mutant strain of *M. tuberculosis* deficient in PknG.

15. The mutant strain of claim 14 comprising a mutated gene encoding PknG.

16. The mutant strain of claim 14 lacking a gene capable of expressing PknG.

17. A method for assessing a difference in virulence of a candidate mycobacterium as compared to a reference mycobacterium, comprising

i) measuring PknG activity in a candidate mycobacterium sample;

ii) comparing the activity determined in (i) to PknG activity in a reference mycobacterium sample, where the reference mycobacterium is of known virulence;

and wherein, a decrease in PknG activity in the candidate sample is indicative of a decrease in virulence as compared to the reference bacterium, and an increase of PknG activity in the candidate sample is indicative of an increase in virulence as compared to the reference bacterium.

18. The method of claim 17 wherein the samples further comprise a PknG substrate.

19. The method of claim 18 wherein the substrate is MPB.

20. The method of claim 18 or 19, wherein activity of PknG is determined by measuring phosphorylation of the substrate.

21. The method of claim 18 or 19, wherein activity of PknG is determined by measuring binding of the substrate to PknG.

22. The method of claim 17, wherein activity of PknG is determined by measuring binding of ATP to PknG.

23. A method of inhibiting mycobacterium PknG activity or survival of a mycobacterium containing PknG, comprising contacting a sample of PknG or a mycobacterium comprising PknG with an isoquinolinesulfonyl compound or salt thereof.

24. The method of claim 23 wherein the method is performed *in vitro*.

25. The method of any one of claims 17-24, wherein the mycobacterium is *M. tuberculosis*.

26. The use of an isoquinolinesulfonyl compound or salt thereof, for preparation of an agent for inhibiting mycobacterium growth or survival.

27. The use of claim 26, wherein the mycobacterium is *M. tuberculosis*.

28. A method of screening for a modulator of a mycobacterium STPK activity comprising:

i) contacting a sample containing a mycobacterium STPK with a candidate agent;

ii) determining activity of STPK in the sample of (i) subsequent to the contacting;

iii) comparing the activity determined in (ii) to activity of the STPK in a sample of (i) without the candidate agent present;

wherein, a difference in STPK activity in samples with and without the candidate agent present, is indicative that the candidate agent modulates STPK activity.

29. An assay for detecting modulation of mycobacterium STPK activity, comprising:

i) determining a first STPK activity level in a sample of a mycobacterial STPK;

ii) determining a second STPK activity level of a sample of (i) further comprising a candidate agent;

iii) comparing the first and second levels;

wherein, difference in the first and second levels relates to an ability of the candidate agent to modulate STPK activity.

30. The method of claim 28 or 29 wherein samples comprising STPK further comprise a STPK substrate.

31. The method of claim 30 wherein the substrate is MPB.

32. The method of claim 30 or 31, wherein activity of STPK is determined by measuring phosphorylation of the substrate.

33. The method of claim 30 or 31, wherein activity of STPK is determined by measuring binding of the substrate to the STPK.

34. The method of claim 28 or 29, wherein activity of STPK is determined by measuring binding of ATP to STPK.

35. The method of any one of claims 28-34, wherein a decrease in STPK activity in the presence of a candidate agent is indicative that the agent will decrease mycobacterial growth or survival.

36. The method of any one of claims 28-35, wherein the STPK is a *M. tuberculosis* STPK.

37. The method of any one of claims 28-36, wherein STPK is present on a solid support.

38. The method of any one of claims 28-37, wherein a STPK substrate is present on a solid support.

39. The method of any one of claims 28-38, wherein a sample without a candidate agent further comprises a STPK inhibitor.

40. The method of claim 39, wherein the inhibitor is an isoquinolinesulfonyl compound or salt thereof.

41. The method of any one of claims 28-40, wherein the STPK is one or more of PknG, PknB, PknJ and PknH.

42. The method of any one of claims 28-40, wherein the STPK comprises PknG.

43. A mutant strain of *M. turberculosis* deficient in one or more STPK selected from: PknG; PknB; PknJ; and PknH.

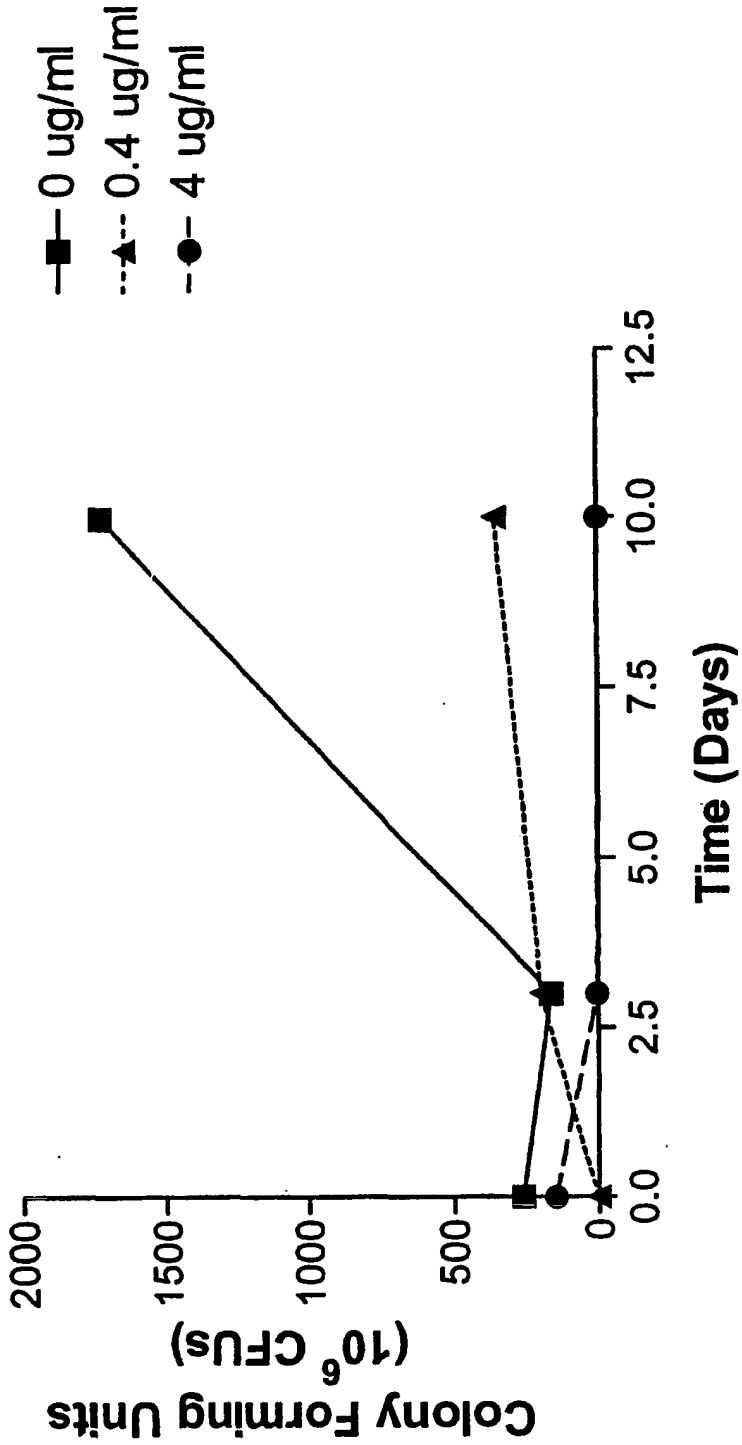
0 ug/ml
0.4 ug/ml
4 ug/ml
Colony Forming Units
(10^6 CFUs)
2000
1500
1000
500
0
0.0
2.5
5.0
7.5
10.0
12.5
Time (Days)

FIG. 1

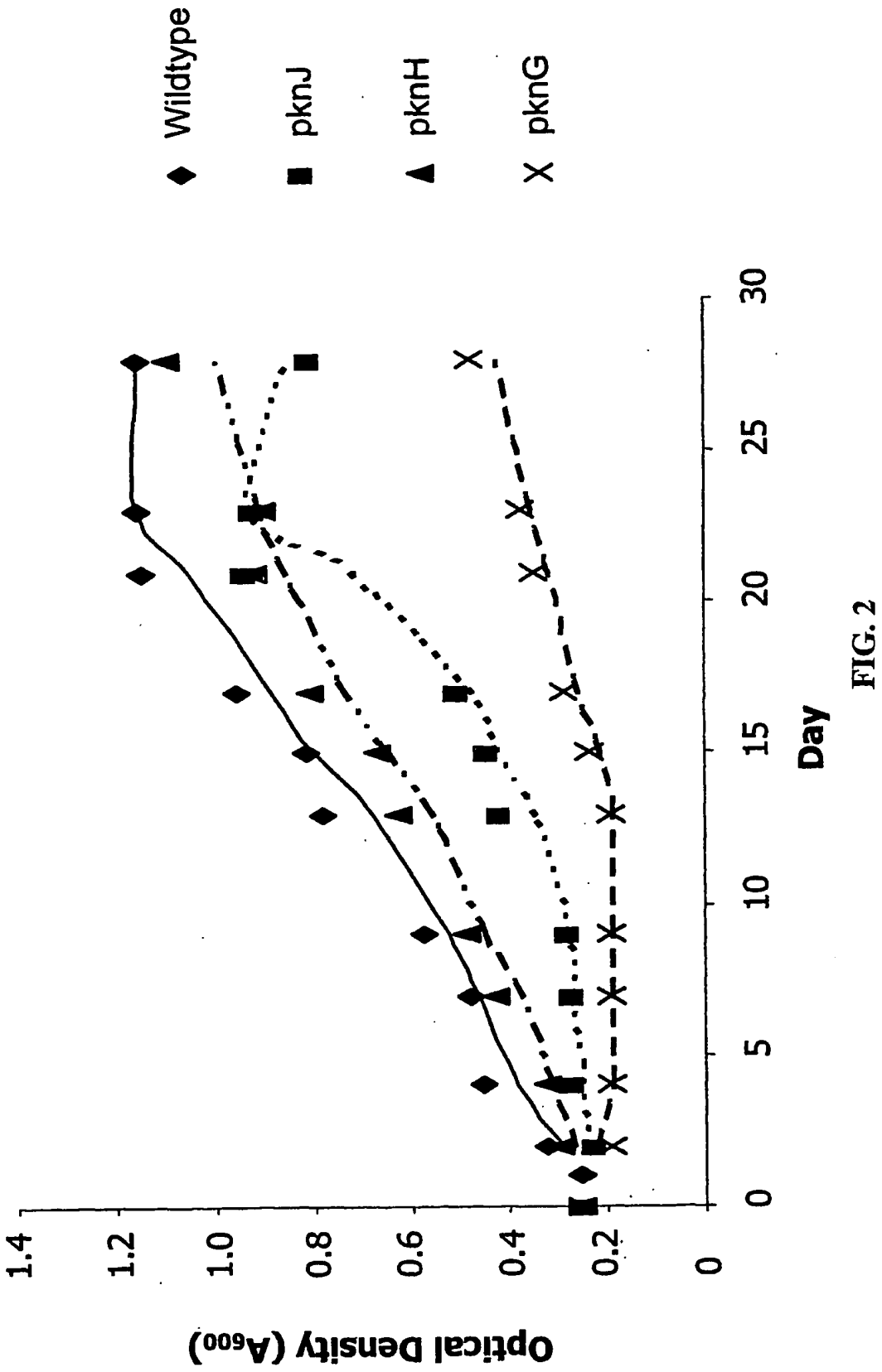
Wildtype
pknJ
pknH
pknG
Optical Density (A_{600})
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
Day

FIG. 2

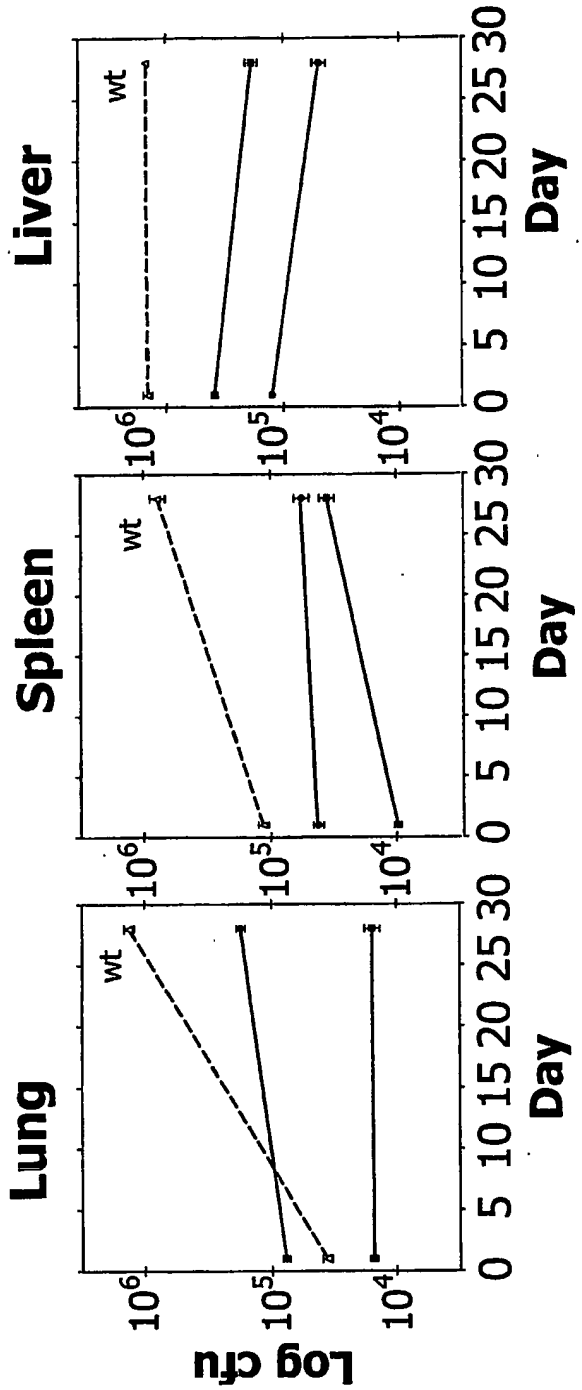

FIG. 3

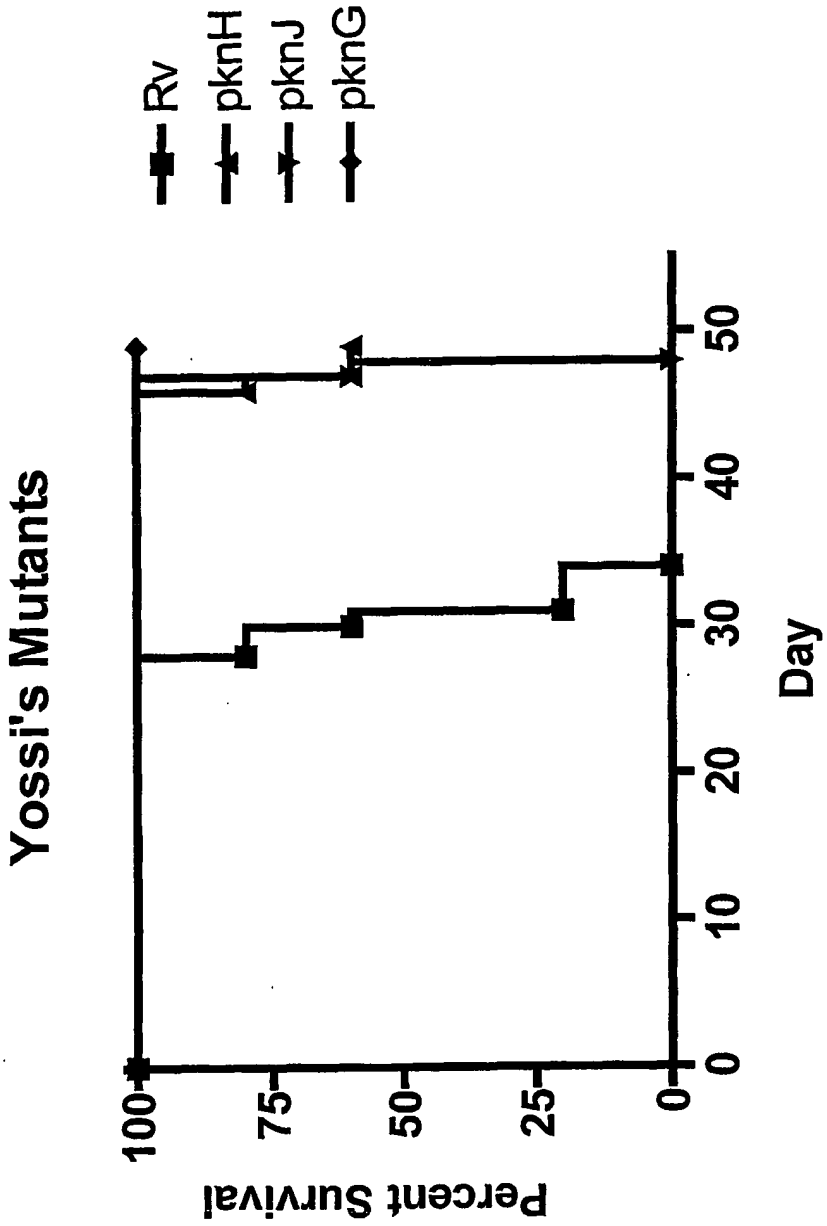
Rv
pknH
pknJ
pknG
Yossi's Mutants
Percent Survival
100
75
50
25
0
0
10
20
30
40
50
Day

FIG. 4

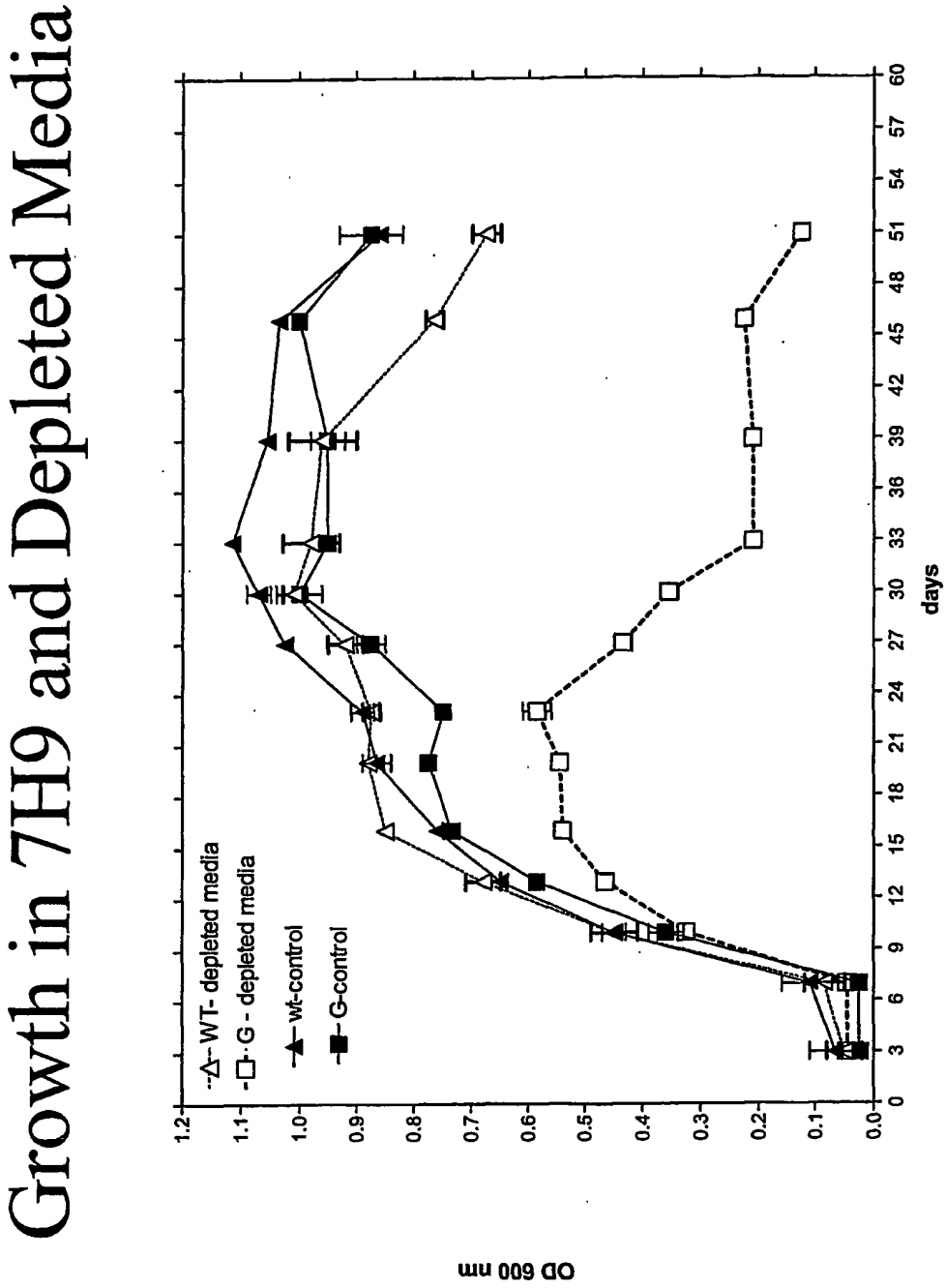
Growth in 7H9 and Depleted Media
OD 600 nm
days
WT- depleted media
G - depleted media
wt-control
G-control
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
0
3
6
9
12
15
18
21
24
27
30
33
36
39
42
45
48
51
54
57
60

FIG. 5

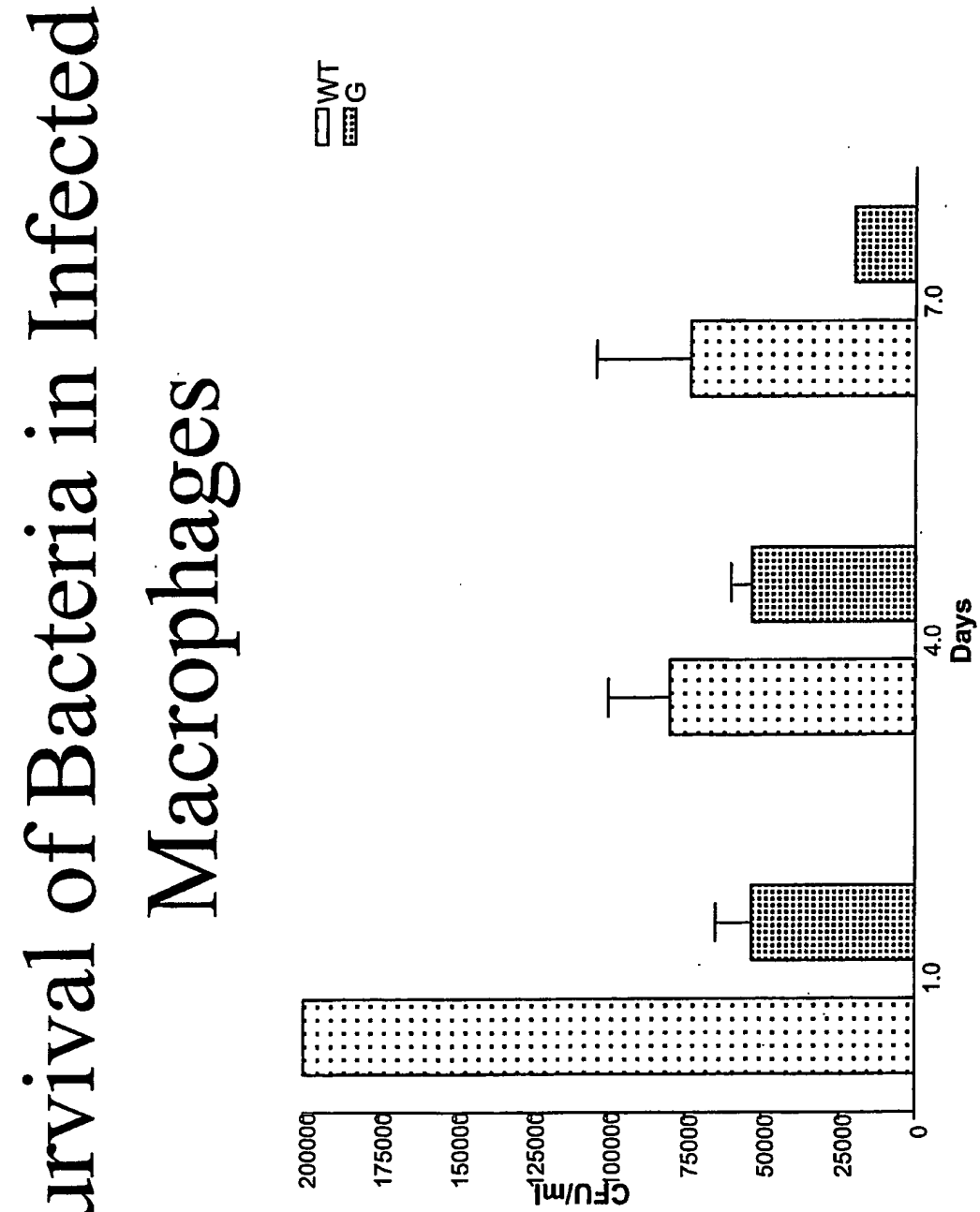
Survival of Bacteria in Infected Macrophages
WT
G
CFU/mL
200000
175000
150000
125000
100000
75000
50000
25000
0
1.0
4.0
7.0
Days

FIG. 6

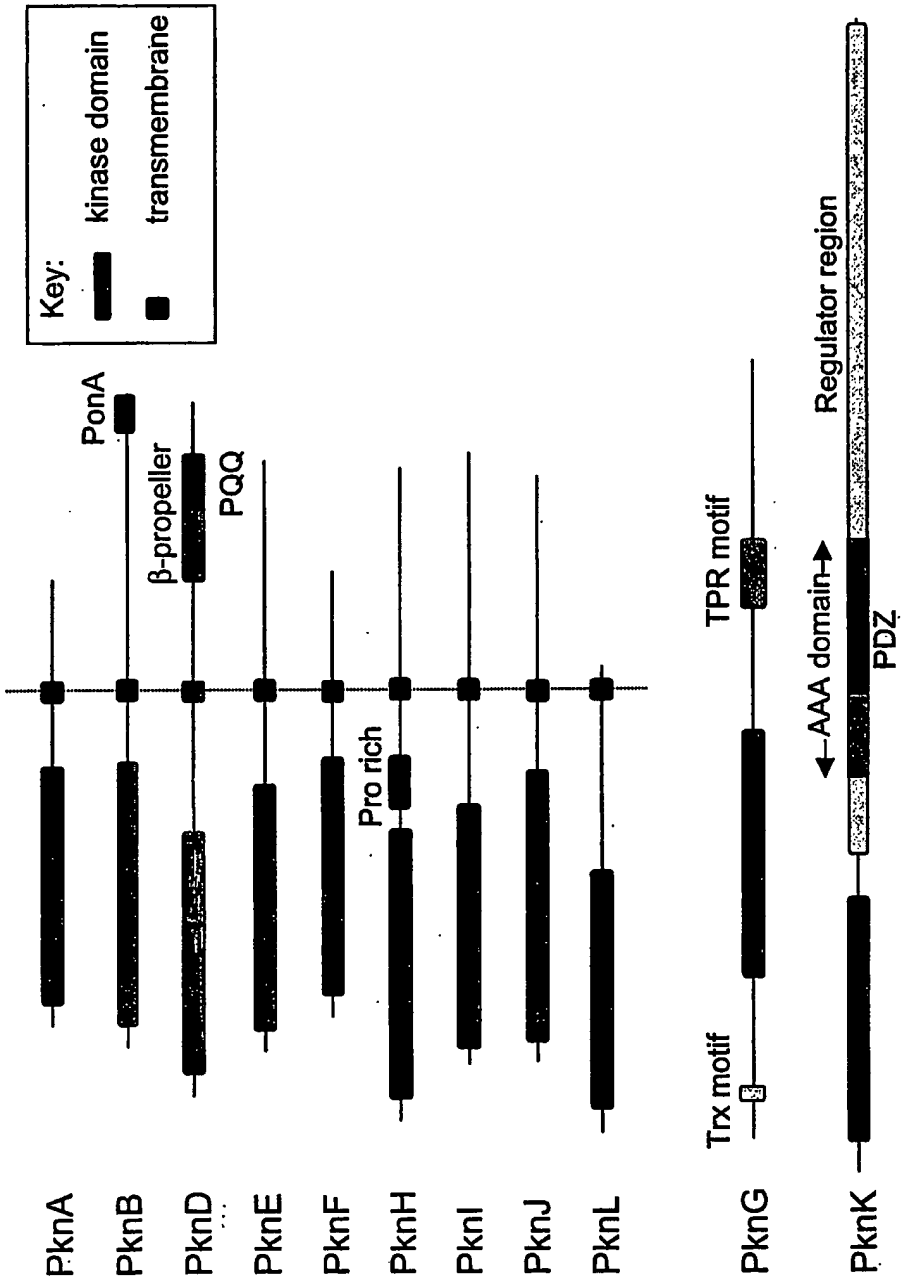
Key:
kinase domain
transmembraine
PonA
β-propeller
PQQ
Pro rich
TPR motif
Trx motif
AAA domain
Regulator region
PDZ
PknA
PknB
PknD
PknE
PknF
PknH
PknI
PknJ
PknL
PknG
PknK

FIG. 7